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Epidemiological Tracing of Bovine Tuberculosis in Switzerland

Multilocus Variable Number of Tandem Repeat Analysis of *Mycobacterium bovis* and *Mycobacterium caprae*

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse Fakultät Universität Zürich

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Zurich, 2015

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1 Summary

Background

After 15 years of absence, in 2013 bovine tuberculosis (bTB) caused by *Mycobacterium* (*M*.) *bovis* and *M*. *caprae* reemerged in the Swiss dairy cattle population.

In order to identify the sources of infection as well as the spreads of the agents, molecular-epidemiologic tracing by MIRU-VNTR analysis in combination with spoligotyping was performed. A total of 17 *M*. *bovis* and 7 *M*. *caprae* isolates were cultured from tuberculous bovine lymph nodes and analyzed with a set of 49 genetic markers by using automated capillary electrophoresis genotyping.

Results

The outbreak in the western part of Switzerland was caused by *M*. *bovis* spoligotype SB0120. With exception of four single-locus variations observed in MIRU 20, the MIRU-VNTR profiles of the 17 *M*. *bovis* isolates were identical, indicating a single source of infection. *M*. *bovis* detected in archival bovine specimens from the outbreak region showed an identical MIRU-VNTR profile, suggesting persistence of the agent in a dairy herd for nearly fifteen years. The outbreak in the eastern part of Switzerland was caused by *M*. *caprae* spoligotype SB0418. All Swiss *M*. *caprae* isolates showed the Lechtal-type MIRU-VNTR profile, described as endemic in wild ruminants and in dairy cattle in Austrian bordering regions. Hence, the agent was most likely introduced by Swiss dairy cattle summering on Austrian pastures.

Conclusions

The present study represents the first MIRU-VNTR analysis of Swiss bTB mycobacterial isolates. The genotyping assay was found to be highly discriminating and suitable for epidemiological tracing of further outbreaks. These findings can contribute to developing a European MIRU-VNTR database to improve the international surveillance of bTB.

Zusammenfassung

Hintergrund

Im März 2013 ist die bovine Tuberkulose (bTB) erneut in der Schweiz ausgebrochen. Zur epidemiologischen Feintypisierung der zwei Pathogene, *Mycobacterium* (*M.*) *bovis* und *M. caprae*, wurde die MIRU-VNTR Analyse kombiniert mit Spoligotyping durchgeführt. 17 *M. bovis* und 7 *M. caprae* Isolate wurden anhand 49 genetischer Marker mittels automatisierter Kapillarelektrophorese analysiert.

Ergebnisse

Der Ausbruch in der Westschweiz wurde durch den *M. bovis* Spoligotyp SB0120 verursacht. Mit der Ausnahme von 4 single-locus Variationen in MIRU 20 waren die MIRU-VNTR Profile der 17 *M. bovis* Isolate identisch, was auf eine einzelne Infektionsquelle hindeutet. Ein *M. bovis* Isolat aus Archivmaterial aus der Ausbruchsregion wies ein identisches MIRU-VNTR Profil auf. Eine asymptomatische Persistenz des Erregers von etwa 15 Jahren in einem Milchkuhbestand konnte deswegen in Betracht gezogen werden. Der zweite Ausbruch wurde in der Ostschweiz detektiert und durch *M. caprae* Spoligotyp SB0418 verursacht. Alle analysierten *M. caprae* Isolate wiesen das MIRU-VNTR Profil vom Lechtaltyp auf, welcher in der Rotwild- und Rinderpopulation in Vorarlberg und Tirol als endemisch beschrieben wurde. Aufgrund dieser Ergebnisse konnte gezeigt werden, dass der Erreger durch Sömmerung in Grenzgebieten eingeschleppt wurde.

Schlussfolgerung

Die vorliegende Untersuchung stellt die erste MIRU-VNTR Analyse von schweizerischen bTB Isolaten dar und zeigt das hohe diskriminative Vermögen dieser Technik zur Genotypisierung weiterer Ausbrüche. Die erhaltenen Erkenntnisse sind ein Beitrag zur Etablierung einer einheitlichen europäischen MIRU-VNTR Datenbank und zur Optimierung der internationalen Überwachung der bTB.

2 Introduction

2.1 The *Mycobacterium tuberculosis* complex

Bovine Tuberculosis (bTB) is a chronic infectious disease caused by bacteria of the *Mycobacterium* (*M.*) *tuberculosis* complex (MTBC). According to the current taxonomy, the following members are grouped within the MTBC: the primarily human pathogens *M. tuberculosis*, *M. africanum* and *M. canettii*; the causative agents of bovine tuberculosis *M. bovis* and *M. caprae*; *M. microti* with a wide range of animal hosts as well as a zoonotic potential; and *M. pinnipedii*, causing tuberculosis in marine mammals [1-4].

M. bovis BCG is another renowned member mentioned among the *M. tuberculosis* complex. In 1908, Albert Calmette and Camille Guérin isolated a virulent *M. bovis* strain from a tuberculous cow in a medium composed of bovine bile, glycerin and potato. Eleven years and about 230 subcultures later they obtained a tubercle bacillus which was not able to induce progressive tuberculosis when injected into guinea pigs, rabbits, cattle and horses. The only licensed vaccine against human tuberculosis since 1921, obtained from *M. bovis* Bacillus Calmette and Guérin (BCG), was developed [5].

Furthermore, two other members of the MTBC have been named after the animal species from which they have been isolated. *M. orygis* was isolated from members of the *Bovidae* family, i.e., oryx antelopes, while the Dassie bacillus was first detected in the lung of a rock hyrax, *Procavia capensis*, also known as “dassie”. None of these novel MTBC members have been found in human Tbc [6].

Recently a new member of the MTBC phylogenetic tree has been isolated from banded mongooses in Botswana and named *M. mungi* [7].

Many studies demonstrate that, despite the different mammalian host tropisms, the MTBC members represent one of the most glaring examples of genetic homogeneity, characterized by 99.9% or greater similarity at genomic level and identical 16S rRNA sequences [1, 2]. Compared to the nucleotide diversity in humans, well-known to be very low, the genomic variety found between *M. tuberculosis* and *M. bovis* strains is on average half as much [8, 9]. Due to the extensive genetic homogeneity between the MTBC members, the definition of ecotypes was suggested to be more appropriate than the classification of species for the different clades [10]. Each ecotype is adapted to a distinct host and can be distinguished by conserved molecular features. Since little evidence for chromosomal DNA exchange such as horizontal gene transfer has been found between MTBC organisms with exception of *M. canettii*, other molecular markers such as deletions or insertions which are known to be conserved among the offspring of a given ecotype and can be used for genetical tracing [10-13].
In this context, Brosch *et al.* computed a phylogenetic tree of the MTBC based on so-called regions of difference (RD). RDs are series of genomic sequences that have been lost from a *M. tuberculosis*- like ancestor and, since such polymorphism cannot be restored by recombination, subsequent evolved clonal populations will all lack these genome sequences. Currently, 20 RDs conserved among members of the MTBC are known [1, 9]. Medically relevant is e.g. RD9, a chromosomal deletion that affects all known animal-adapted members of the MTBC [1]. Thus, the presence or absence of RD9 enables to differentiate animal tuberculous mycobacteria from the human pathogens *M. tuberculosis* and *M. canettii* as shown in **Fig.1**.

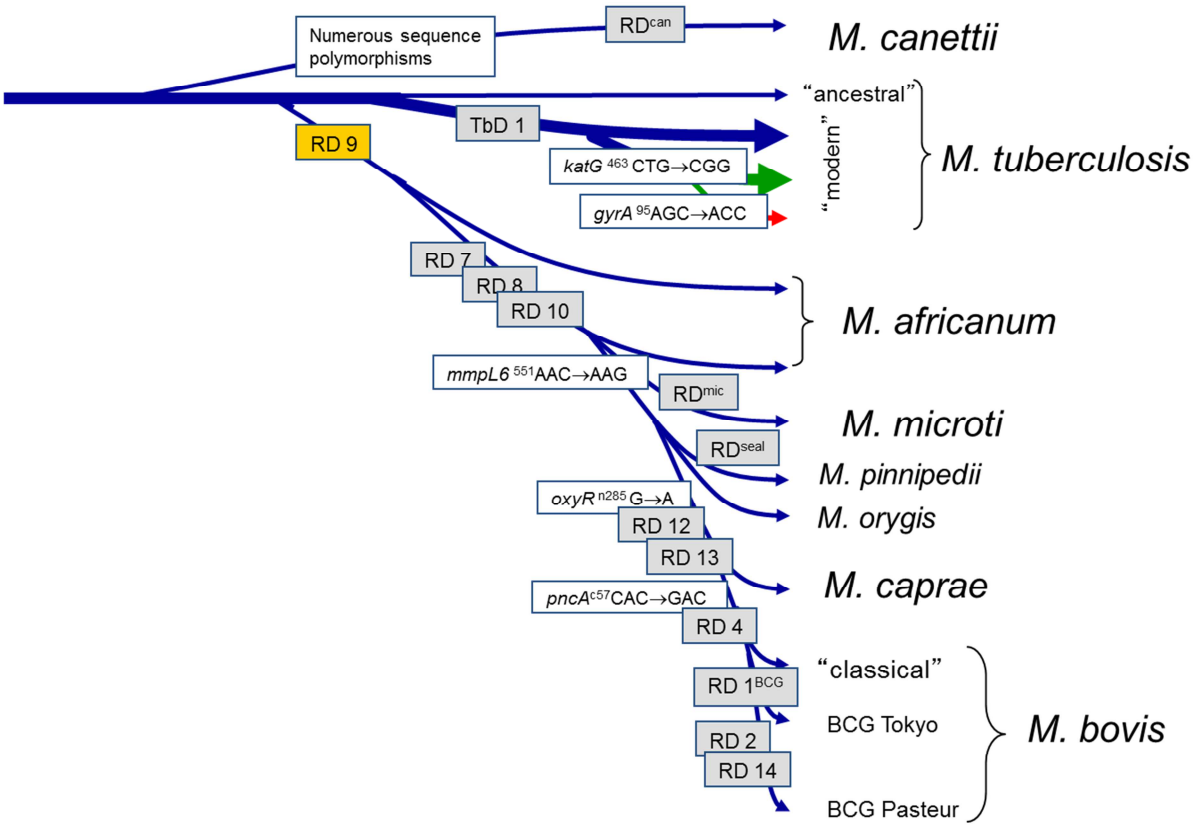

Figure 1. Clonal evolution of the *Mycobacterium tuberculosis* complex modified from Brosch *et al.*. Region of difference 9 (RD9) mentioned above in text is displayed in yellow.

2.2 Bovine Tuberculosis

To date, the most relevant members of the MTBC as a cause of bovine tuberculosis in Europe are *M. bovis* and *M. caprae*. Both species can also affect wild and other domestic animals as well as human beings. Several studies point out that the two mycobacterial species cause similar macroscopic lesions in humans [14, 15]. In contrast, recent findings in wild boars (*Sus scrofa*) demonstrated significant histopathological differences between *M. bovis*- and *M. caprae*-infected animals. A presence of numerous granulomas in advanced stage, a significantly higher number of acid-fast bacilli as well as counts of multinucleated giant cells could be observed in animals infected by *M. caprae* compared with those infected by *M. bovis*. These results suggest a greater tendency towards excretion and therefore a higher risk of transmission of *M. caprae* to other individuals [16]. In cattle, significant morphological differences of gross and histopathological lesions induced by *M. bovis* or *M. caprae* have not been described yet [17, 18].

The zoonotic potential of bTB has been largely described; direct contact with tuberculous cattle, inhalation of aerosols or consumption of contaminated uncooked products, e.g. raw milk, are the most common ways of transmission [19-22]. The isolation of *M. tuberculosis*, the agent of human tuberculosis, in cattle has been reported in central European countries, Russia, the USA and Japan [10, 23-25]. In Switzerland, during the final stage of the eradication of bTB, sporadic cases of *M. tuberculosis* infections in cattle were identified and epidemiologically traced back to humans with extended pulmonary tuberculosis, e.g. the owners, members of their families or farmhands [26].
Infection in cattle by *M. tuberculosis* does not induce active tuberculosis in cattle but causes a strong cell-mediated immune response, which can lead to a positive intradermal comparative cervical tuberculin test indistinguishable from *M. bovis*-infected cows [12].

M. tuberculosis is known to persist in cattle with low loads of bacilli, but it is not able to cause any pathological signs of disease in the infected animals [12, 27]. More recently, three cases of tuberculosis in cattle caused by *M. tuberculosis* were identified in Spain. In all cases, farmworkers suffering from active tuberculosis were identified as the source of infection: human and animal isolates shared identical MIRU-VNTR patterns and belonged to the same spoligotype [28].

2.2.1 Bovine tuberculosis in Switzerland

A first attempt for a nationwide disease control program was started in 1896. The Swiss government placed tuberculin tests at the disposal of the cantonal veterinary offices and provided financial support for voluntary farm testing [29].
In the 1930's, the Swiss Federal Council decided to intensify the tuberculosis control measurements with the aim of eradicating the disease from farm animals [30]. It immediately turned out as a difficult project due to the high prevalence of up to 50% in some dairy herds and the scarcity of information about the wild animal status [31].
After more than a decade of different cantonal bTB control approaches with the aim of minimizing the economic losses, in March 1950 a federal law for the eradication of bTB was approved. This procedure was based on the successful eradication model of the USA and consisted in the culling of all tuberculin skin reactor animals. Nine years later, on the 15th of December 1959, Switzerland was officially declared free from bTB according to the requirements of section 3.2.3.10 of the OIE Animal Health Code [32]. During this eradication program, based on a nationwide test-and-slaughter concept with costs of about 400 million Swiss francs, approximatively 25% of the Swiss cattle population was culled [30].
Subsequently, the disease surveillance consisted in the meat inspection at abattoir level combined with comparative intradermal tuberculin tests on imported livestock. In the past 50 years, only sporadic within-one-herd outbreaks of bTB were registered. However, in March 2013 an outbreak involving nine dairy farms in the western region of Switzerland was detected. The causing agent has been identified as *M. bovis.* Few months later, in November 2013, as a result of the more intensive nationwide monitoring program, a new outbreak in the eastern region of the country was recognized. The causative agent was *M. caprae*. Switzerland's new confrontation with bovine tuberculosis in 2013 had an important political and economic impact. Especially for export-oriented sectors of the Swiss dairy production, a reemergence of bTB would result in considerable economic problems. A disease that had been considered to belong to the past or to be confined to other countries, suddenly emerged again and alarmed farmers, veterinary authorities and consumers. Preventing the spread of the disease has been defined as the highest priority. Within this purpose, the understanding of the pathogen's provenience, in order to avoid further infections, appeared fundamental. Also owing to Switzerland's strong international connections, internationally competitive state-of-the-art diagnostic tools were and still are necessary for the monitoring and epidemiological tracing of bTB in Switzerland.

2.3 Molecular typing of *M. tuberculosis* complex bacteria

Spoligotyping

Spoligotyping (spacer oligotyping) is a widely used PCR-based method that through different hybridization patterns proves the presence or absence of 43 target sequences in the direct repeat (DR) region in strains of the *M. tuberculosis* complex. The DR consists of multiple repeated sequences of 36 base pairs interspersed with non-repetitive spacers of similar size (direct variant repeat). Spacers' sequences are specific to the DR region and copies could not be found elsewhere in the genome. The loss of those sequences, probably resulting from homologous recombination of DRs and excision of the recombined fragment, occurs during DNA replication. The lost spacers cannot be substituted or regained, resulting in a unidirectional evolution. In fact, more than 60 spacer units have been described in the DR region, but only 43 were selected for the internationally adopted technique. To the author's knowledge, the order of the 43 spacers in the genome is well conserved; meanwhile the function of the described locus in *M. tuberculosis* is still undisclosed [33, 34]. Even if more elaborate analysis targets, such as single nucleotide polymorphism (SNPs) or large sequence polymorphisms are to date available, spoligotyping remains an essential tool in veterinary mycobacteriology. The reason for this is the fact that it allows the identification of individual mycobacterial strains and, thus, the clarification of the dynamics of transmission among livestock or between wild and domestic animals in a cost-effective way [1, 12, 35].

Insertion Sequence 6110 – Restriction Fragment Length Polymorphism

Insertion Sequence (IS) 6110 is an extensively adopted criterion for differentiating *M. tuberculosis* complex members. The variable locations in the genome and the number of present copies are generally analyzed by the restriction fragment length polymorphism (RFLP) technique [36].
The main disadvantage of RFLP is the relatively long timespan needed between sample collection and result report. Often weeks up to months are required to reach the growth level necessary for analysis. Moreover, another weakness of the RFLP technique is the insufficient discrimination of strains with a low number of IS6110 copies, such as *M. bovis* strains.

Multilocus Variable Number of Tandem Repeat Analysis

Using complete genome sequencing of various members of the MTB complex, a novel class of genetic markers has been identified [11, 37]. First of all, Exact Tandem Repeats (ETRs) were described [38], subsequently, other relevant gene loci were recognized for molecular typing: Mycobacterial Interspersed Repetitive Units (MIRUs) [39], Variable Number Tandem Repeats (VNTRs) [40, 41], and Queen's University Belfast (QUB)-VNTRs [42].
Multilocus variable number of tandem repeat analysis (MLVA) is a genotyping method based on PCR amplification using oligonucleotide primers targeting the flanking regions of polymorphic gene loci. The amplicons' size is then determined by electrophoretic migration, and since the length of each repeat unit for the different loci is known, the number of the amplified MIRU-VNTR copies can be calculated. The obtained numerical code corresponds to the number of repeat units present in each analyzed locus and permits an accurate isolate identification. The repetitive units of the most used MIRU-VNTRs are classified as minisatellites, since their size typically ranges from 10 to 100 base pairs [39].

Multiple sets of informative VNTR markers were described for *M. tuberculosis* [39, 40, 43-45] and also for *M. bovis* [46-48]. Many of them have shown a high discriminatory power but their application remains restricted to mycobacterial samples from a given geographical area.
Hence, many authors have emphasized the necessity of defining discriminatory panels of gene loci for different mycobacterial species, which are known to exhibit genetical variability in different geographical regions [35, 46, 48-50].
In the recent past more elaborate techniques such as single nucleotide polymorphism (SNPs) [12] and large sequence polymorphism [51] have been developed to better differentiate mycobacterial strains. Within this context it should be highlighted that with such a wide range of DNA typing methods it has become even more difficult to make use of the appropriate technique. The lack of an international data-base that permits comparison of genotyping findings and the need of having straightforward, rapid and cost-effective techniques make these new techniques unattractive for epidemiological purposes. The MIRU-VNTR method meets the mentioned requirements and additionally provides a satisfactory degree of discrimination and stability. To date, the highest discriminatory power of the MTB complex members can be achieved by using multiple MIRU-VNTR markers, including the commonly named hypervariable loci, combined with spoligotyping [47, 52, 53].
For this reason, in order to characterize the isolates originating from the Swiss outbreaks, the MIRU-VNTR and spoligotyping techniques were chosen.

2.4 Aims of the present study

The purpose of the present study was to genotype the *M. bovis* and *M. caprae* strains isolated from the two recent outbreaks of bovine tuberculosis which occurred in Switzerland using the MIRU-VNTR methods combined with spoligotyping.
Additionally, this study aimed to establish a high-throughput automated procedure enabling epidemiological tracing of tuberculous mycobacteria from Swiss cattle.

3 Material and Methods

3.1.1 Ethical statement

As part of the nationwide Swiss meat inspection program (817.190 Ordinance of 23 November 2005 on the Slaughter of Animals and Meat Inspection), every slaughtered ungulate has to be controlled for epizootic diseases and in particular for zoonotic agents. Lymph nodes showing suspicious lesions for bovine tuberculosis have to be sent to the National Reference Laboratory of Veterinary Bacteriology (IVB), University of Zurich.
The obtained samples are first macroscopically evaluated by qualified personnel and any alteration is routinely recorded. Lymphatic tissue of different nodes is subsequently pooled per animal for culture and PCR. The standardized protocol for direct detection of the pathogen consists in microscopic identification of acid-fast bacilli (Ziehl-Neelsen stain), a specific Real-Time PCR (artus® , *M. tuberculosis* TM PCR Kit, Qiagen GmbH, Hilden, Germany) and culture on three different media (Technische Weisungen über die Untersuchungen auf bovine Tuberkulose, 27. September 2010).
Since suspicious samples are submitted at the IVB as part of the Swiss national bTB surveillance program, no animals had to be specifically killed for this research project. An ethical approval was not necessary.

3.1.2 Sample collection

Lymphatic tissue analyzed in this study has been collected at the National Reference Laboratory of Veterinary Bacteriology, University of Zürich from March 2013 until November 2014. In total, we received samples from 528 animals of the species *Bos taurus primigenius* sent from all Swiss cantons. On average, we took delivery of three lymph nodes for each animal with a minimum of one and a maximum of five. In most cases, collected lymph nodes were of retropharyngeal, mandibular, bronchial or mediastinal origin. Furthermore, suspicious materials as caseous liver abscesses, granulomatous lung, pleura or mesenteric alteration were examined.

3.1.3 Mycobacterial culture

Approximatively 2 grams of tissue specimen were homogenized in 20 mL 0.15 M NaCl by using a rotating-blade macerator system (T 18 Digital Ultra-Turrax IKA, Staufen, Germany) and centrifuged at 3000 x g for 15 min. The supernatant was decanted and the deposit was decontaminated according to De Kantor and co-workers with slight modifications [54]. Briefly, the sediment was thoroughly resuspended in 4.0 mL 4.0 M H_2SO_4, incubated at room temperature for 15 min, and neutralized by adding 5.65 mL 1 M NaOH. Afterwards, 20 mL 0.15 M phosphate buffered saline solution (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) were added and the suspension was centrifuged at 3000 x g for 15 min. The final sediment was resuspended in 2.5 mL PBS and used as inoculum. Two aliquots of 0.25 mL each were inoculated on an agar slant of Lowenstein-Jensen medium (BBL™Lowenstein-Jensen™ TB Medium + PACT, Becton and Dickinson Company, Franklin Lakes, New Jersey, United States) and a Stonebrink medium (BBL™Stonebrink™ TB Medium + PACT, Becton and Dickinson Company, Franklin Lakes, New Jersey, United States). Inoculated slants were incubated at 37°C ± 1.5°C and examined weekly.

From the suspension, 0.5 ml were inoculated into a liquid culture medium MGIT™ (Mycobacteria Growth Indicator Tube; BD BACTEC™ MGIT™ + OADC Enrichment + PANTA™ Antibiotic supplement, Becton and Dickinson Company, Franklin Lakes, New Jersey, United States). As proposed by Gormley *et al.* [55, 56], we enriched the liquid culture broth by adding 100 µl sodium-pyruvate (4.2 g/L) (Sigma-Aldrich, Buchs, Switzerland) to enhance the growth of *M. bovis* and *M. caprae*. All samples were incubated for seven weeks in a BACTEC™ MGIT™ 320 incubator (Becton and Dickinson Company, Franklin Lakes, New Jersey, United States). BACTEC™ MGIT™ 320 incubator automatically detects an oxygen consumption through a fluorescent compound (Ruthenium-Komplex) embedded in silicone on the bottom of the MGIT™, leading to a rapid and reliable identification of growing aerobic bacteria. Positive cultures were microscopically examined for the presence of acid-fast bacilli after staining smears with Ziehl-Neelsen stain.

The entire procedure was performed in a laminar flow cabinet by qualified personnel at the Institute of Veterinary Bacteriology, University of Zurich. Incubation and controls of cultures for mycobacterial growth were performed under biosafety level 3 (BSL3) conditions in the same Institute as previously mentioned.

3.1.4 Extraction of genomic DNA from mycobacteria

A loop-full of colony material obtained from liquid culture medium MGIT™ was suspended in 180 µl ATL buffer (Qiagen GmbH, Hilden, Germany) into a 2mL microtube containing ceramic beads (1.4 mm ø, Omni International, Kennesaw, USA). The mixture was heated at 99 °C for 30 min and subsequently homogenized through bead beating. Four cycles of 45 seconds each (6,500 rpm) were performed in order to release mycobacterial DNA (Precellys® 24 homogenizer. Bertin Technologies, Montigny, France). Enzymatic protease digestion was performed by adding 20 µl of Proteinase K (Qiagen GmbH, Hilden, Germany) and incubation at 56 °C for 12 h under constant shaking (BioShake IQ, Analytik Jena AG, Germany). DNA purification has been completed using the QIAamp cador® Pathogen Mini Kit automated program performed by the QIAcube® instrument (Qiagen GmbH, Hilden, Germany). A specific Real-Time PCR (artus® , *M. tuberculosis* TM PCR Kit, Qiagen GmbH, Hilden, Germany) was performed for each purified sample in order to assure the presence of DNA from a member of the *Mycobacterium tuberculosis* complex. DNA concentration was afterwards determined using NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific Inc.) and if necessary diluted by adding molecular grade water (Milli-Q Integral 3, Millipore Corporation) to achieve a final concentration of 100 pg/µl. The obtained specimens were afterwards stored at –20 °C until further molecular investigation.

Three *M. tuberculosis* complex reference strains, *M. tuberculosis* H37Rv, *M. bovis* BCG Tice ATCC 27289™ and *M. bovis* BCG Pasteur ATCC 35734™ were included as reference isolates and positive controls.

3.1.5 Genotyping methods

Reversed line blotting

In order to determine the species among the positive isolates, every positive culture was tested with GenoType® MTBC kit (HainLifescience GmbH, Nehren, Germany) according to the manufacturer's protocol.

Spoligotyping

Spoligotype analysis was performed by the French Agency for Food, Environmental & Occupational Health Safety (ANSES), Maisons-Alfort Cedex, as described by Kamerbeek *et al*. [57]. Spoligotype patterns had been assigned according to the international nomenclature [58].

3.2 MIRU-VNTR analysis

3.2.1 VNTR loci identification and PCR conditions

The genotyping analysis has been started based on 24 loci showing variable numbers of tandem repeat units as proposed by Supply *et al.* 2006 for *M. tuberculosis* [35]. To date, this technique is considered the reference method for investigation of *M. tuberculosis* transmission chains in human medicine [56]. The only modification to the standard technique was locus VNTR 2461 (ETR-B) for which the primer pairs proposed by Frothingham [38] were used as shown in **Table 1**.

Table 1. PCR primer sequences and characteristics of the 24 standard panel.

Marker	Alias	Primer pair sequence, 5' – 3'	Amplicon size (bp) in H37Rv	Repeat no. x length (bp) in H37Rv	Primer reference
154	MIRU 02	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT	508	2 x 53	(Supply et al., 2006) (Supply et al., 2006)
424	Mtub04	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC	639	2 x 51	(Supply et al., 2006) (Supply et al., 2006)
577	ETRC	CGAGAGTGGCAGTGGCGGTTATCT AATGACTTGAACGCGCAAATTGTGA	382	4 x 58	(Supply et al., 2006) (Supply et al., 2006)
580	MIRU 04 / ETRD	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC	353	3' x 77	(Supply et al., 2006) (Supply et al., 2006)
802	MIRU 40	GGGTTGCTGGATGACAACGTGT GGGTGATCTCGGCGAAATCAGATA	408	1 x 54	(Supply et al., 2006) (Supply et al., 2006)
960	MIRU 10	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT	643	3 x 53	(Supply et al., 2006) (Supply et al., 2006)
1644	MIRU 16	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC	671	2 x 53	(Supply et al., 2006) (Supply et al., 2006)
1955	Mtub21	AGATCCCAGTTGTCGTCGTC CAACATCGCCTGGTTCTGTA	206	2 x 57	(Supply et al., 2006) (Supply et al., 2006)
2059	MIRU 20	TCGGAGAGATGCCCTTCGAGTTAG GGAGACCGCGACCAGGTACTTGTA	591	2 x 77	(Supply et al., 2006) (Supply et al., 2006)
2163b	QUB11b	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT	412	5 x 69	(Supply et al., 2006) (Supply et al., 2006)
2165	ETRA	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT	420	3 x 75	(Supply et al., 2006) (Supply et al., 2006)
2347	Mtub29	GCCAGCCGCCGTGCATAAACCT AGCCACCCGGTGTGCCTTGTATGAC	563	4 x 57	(Supply et al., 2006) (Supply et al., 2006)
2401	Mtub30	CTTGAAGCCCCGGTCTCATCTGT ACTTGAACCCCCACGCCCATTAGTA	363	2 x 58	(Supply et al., 2006) (Supply et al., 2006)
2461	ETRB	ATGGCCACCCGATACCGCTTCAGT CGACGGGCCATCTTGGATCAGCTAC	292	3 x 57	(Frothingham & Meeker-O'Connell, 1998) (Frothingham & Meeker-O'Connell, 1998)
2531	MIRU 23	CTGTCGATGGCCGCAACAAAACG AGCTCAACGGGTTCGCCCTTTTGTC	465	6 x 53	(Supply et al., 2006) (Supply et al., 2006)
2687	MIRU 24	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA	447	1 x 54	(Supply et al., 2006) (Supply et al., 2006)
2996	MIRU 26	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG	438	3 x 51	(Supply et al., 2006) (Supply et al., 2006)
3007	MIRU 27	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA	657	3 x 53	(Supply et al., 2006) (Supply et al., 2006)
3171	Mtub34	GGTGCGCACCTGCTCCAGATAA GGCTCTCATTGCTGGAGGGTTGTAC	488	3 x 54	(Supply et al., 2006) (Supply et al., 2006)
3192	MIRU 31 / ETRE	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT	651	3 x 53	(Supply et al., 2006) (Supply et al., 2006)
3690	Mtub39	CGGTGGAGGCGATGAACGTCTTC TAGAGCGGCACGGGGGAAAGCTTAG	562	5 x 58	(Supply et al., 2006) (Supply et al., 2006)
4052	QUB26	AACGCTCAGCTGTCGGAT CGGCCGTGCCGGCCAGGTCCTTCCCGAT	708	5 x 111	(Supply et al., 2006) (Supply et al., 2006)
4156	QUB4156	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT	681	2 x 59	(Supply et al., 2006) (Supply et al., 2006)
4348	MIRU 39	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT	646	2 x 53	(Supply et al., 2006) (Supply et al., 2006)

The panel of tested loci was then extended with 25 additional MIRU-VNTR markers found by literature research (PubMed, http://www.ncbi.nlm.nih.gov/pubmed/ last accessed, 13.09.2014). Six of them, VNTR 3336 [59], VNTR 1451 [41], VNTR 1612 [42], VNTR 0024, VNTR 3663 and VNTR 2990 [40], were subsequently excluded because of unsatisfactory PCR amplification. The panel was finally enriched (**Table 2**) with another set of six loci designed by the author using the Tandem Repeats Finder web site (http://tandem.bu.edu/trf/trf.html (last accessed, 13.09.2014)). The described web site permitted the identification of tandem repeats showing different lengths by *in-silico* analysis of *M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur 1173P2.

The newly described loci were named following the VNTR nomenclature convention proposed by Smittipat *et al.* [45], based on the first four of seven digits of the nucleotide number in the genome of the H37Rv strain. In the same way as for the 24 loci conventional panel, we set primers of 17-24 bp in the flanking region of the VNTR loci. Allele determination was performed by sizing the PCR products and was based on the H37Rv complete genome sequence available on Pubmed, http://www.ncbi.nlm.nih.gov/nuccore/444893469?report=fasta (last accessed, 13.09.2014).

Table 2. PCR primer sequences and characteristics of the 31 additional MIRU-VNTR analyzed loci**.**

Marker	Alias	Primer pair sequence, 5' – 3'	Amplicon size (bp) in H37Rv	Repeat no. X length (bp) in H37Rv	Primer reference
0024	Mtub01	GACAAACAGGAGGGCGTTG TATTACGACGACCGCTATGC	328	10 x 18	(Le Fleche et al., 2002) (Le Fleche et al., 2002)
0079	Mtub02	CGTGCACAGTTGGGTGTTTA TTCGTTCAGGAACTCCAAGG	230	5 x 6	(Le Fleche et al., 2002) (Le Fleche et al., 2002)
569		TCAGCGTGTTTGTTACCC CTCTCGCCCATACCGGA	632	2 x 56	(Smittipat et al., 2005) (Smittipat et al., 2005)
917		CTTCGACTGTTCCAGCTGAC TATCTTCGAGCGCGAGGACT	436	2 x 58	(Smittipat et al., 2005) (Smittipat et al., 2005)
1121	Mtub12	CTCCCACACCCAGGACAC CGGCCTACCCAACATTCC	215	3 x 15	(Le Fleche et al., 2002) (Le Fleche et al., 2002)
1281c	QUB1281c	GGGGCTGCGGACCTACGGACT CTGGACTCTTGCGGGGACTTCG	184	2 x 60	(Roring et al., 2002) (Roring et al., 2002)
1305		TGGAGGAGACCATCTCGC CCTTGTCGAGTCGGTTGC	344	2 x 62	(Smittipat et al., 2005) (Smittipat et al., 2005)
1443		CTTCGGCGACCTGTGGAATG GTAGCGGTGCAAACCCAAATTG	338	1 x 56	This study This study
1451	QUB1451	GGTAGCCGTCGTCGAGAAGC CGCCACCACCGCACTGGC	305	3 x 57	(Roring et al., 2002) (Roring et al., 2002)
1612	QUB23	GCTGCACCGGTGCCCATC CACCGGAGCCGGAACGGC	141	5 x 21	(Skuce et al., 2002) (Skuce et al., 2002)
1895	QUB1895	GGTGCACGGCCTCGGCTCC AAGCCCCGCCGCCAATCAA	319	4 x 57	(Roring et al., 2002) (Roring et al., 2002)
1907		GAACGTTGGAAGAGATCAGCC TACATCGGTACGCTCTCAACG	589	2 x 56	(Smittipat et al., 2005) (Smittipat et al., 2005)
1982a		GGAATTGAAGAAGCCCGACGAAG CATCAACTCGATCGGCGTCG	379	2 x 75	This study This study
1982	QUB18	CCGGAATCTGCAATGGCGGCAAATTAAAAG TGATCTGACTCTGCCGCCGCTGCAAATA	620	5 x 78	(Supply et al., 2006) (Supply et al., 2006)
2074	Mtub24	AAATTCAAAGAGTTTCTCGACAGTG GATCTTGAGAACCAAGATGTCCTT	805	3 x 56	(Le Fleche et al., 2002) (Le Fleche et al., 2002)
2163a	QUB-11a	CCCATCCCGCTTAGCACATTCGTA TTCAGGGGGGATCCGGGA	305	2 x 69	(Skuce et al., 2002) (Skuce et al., 2002)
2372		ACCTCCGTTCCGATAATC CAGCTTTCAGCCTCCACA	298	2 x 57	(Smittipat et al., 2005) (Smittipat et al., 2005)
2705		CACGGTCTTGCCCAGCAAAC GTTTGCTGGGCAAGACCGTG	229	1 x 94	This study This study
2990	Mtub31	GTGACGTTTACCGTGCTCTATTTC GTCGTCGGACAGTTCTAGCTTT	257	2 x 55	(Le Fleche et al., 2002) (Le Fleche et al., 2002)
3155	QUB15	AGGGGTTCTCGGTCACCC TACATTCGCGGCCAAAGG	252	3 x 54	(Supply et al., 2006) (Supply et al., 2006)
3189		CCGTGACGGTTCAGTACATCC GTTTCCTGCACGGTACGAGAC	217	2 x 57	This study This study
3232	QUB3232	CGGCGATGGTGCCGCCATG CTTGGTGAAGGCCCCGATG	349	3 x 56	(Supply, 2006) (Supply, 2006)
3239	ETR F	CTCGGTGATGGTCCGGCCGGTCAC GGAAGTGCTCGACAACGCCATGCC	476	2 x 79	Frothingham & Meeker-O'Connell, 1998) Frothingham & Meeker-O'Connell, 1998)
3291		TGCGCTCACTACCTCGATTGTC GAGTATGTCGCCCAGCAAATGTC	217	2 x 57	This study This study
3351		CGACCGATACGCATTTGGATGAG GAAACGAGCTTGCGGTCACC	290	2 x 56	This study This study
3336	QUB3636	GATCGGGTGCAGTGGTTTCAGGTG GGGCGGCCAGCGGTGTC	571	5 x 59	(Allix-Beguec et al., 2014) (Allix-Beguec et al., 2014)
3594		ACCAGTACGAACCAACCTGC AACCGTGAGCTGAAGGCG	498	3 x 56	(Smittipat et al., 2005) (Smittipat et al., 2005)
3663	Mtub38	GCCCAAAAAGCATGGGAACGTGCCCCT GGTTGTCCCCGCAGTATCTC	373	4 x 63	(Le Fleche et al., 2002) (Le Fleche et al., 2002)
3820		ACCCGTAAGCCGCTGCTG CCAGGTAGACGCAGACGCG	310	3 x 57	This study This study
4120		GTTCACCGGAGCCAACC GAGGTGGTTTCGTGGTCG	447	2 x 57	(Smittipat et al., 2005) (Smittipat et al., 2005)
4155		GACTACTACGAGCACGCGATG GTGATTGACGGTGCGATACCG	227	2 x 57	This study This study

The PCR reaction mixture for the tested loci was composed of 1 x HotStar *Taq* Master Mix Kit (Qiagen GmbH, Hilden, Germany), 1x Q-Solution® (Qiagen GmbH, Hilden, Germany), a 0.5 µM concentration of the primer pairs and approximately 200 pg purified mycobacterial DNA in a final volume of 10 µl. The reaction was performed with a Veriti® Thermal Cycler (Applied Biosystems®) starting from a *Taq* Polymerase activation step of 15 min at 95 °C. After activation, the PCR was performed for 40 cycles of 1 min at 94 °C, 1 min at 59 °C and 1 min and 30 sec at 72 °C. The PCR cycles were concluded by an incubation of 10 min at 72 °C.

3.2.2 Capillary electrophoresis

PCR products were analyzed using a capillary electrophoresis device (QIAxcel. Qiagen GmbH, Hilden, Germany). In order to assess the fragment sizes of the tested loci we analyzed 10 µl of PCR- product with a QX DNA high-resolution cartridge, using the OH1700 AM10sec method and QX 15 bp – 3 kb alignment marker. In our case the most accurate size marker enabling an accurate fragment size determination was QX 100bp – 2.5 kb. The results were exported in either the gel-overview (**Figure 2**) or electropherogram format (**Figures 4-7**) and analyzed with the QIAxcel ScreenGel Software (Qiagen GmbH, Hilden, Germany).

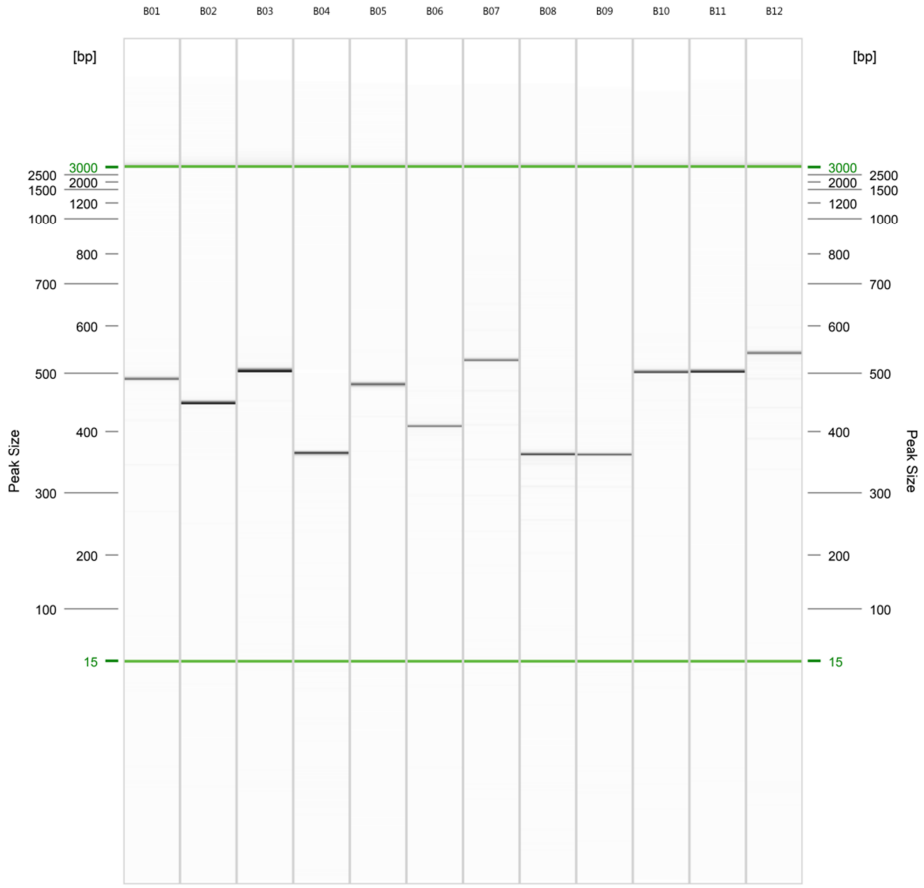

Figure 2. Gel overview exported from QIAxcel ScreenGel Software showing three strains tested on four MIRU-VNTR loci. Recognizable are the different fragment sizes present in two loci (B01-B03 and B04-B06) resulting in 3 distinct alleles for each strain. In contrast, the strains at position B08-B09 and B10-B11 share the same allele. The alignment marker bands (QX 15 bp – 3 Kb) are shown in green.

3.2.3 Confirmation of results accuracy in case of double bands

The fragments were separated by conventional gel electrophoresis in a 1% pulsed field certified agarose (Bio-Rad Laboratories, Cressier, Switzerland), stained with UltraPure™ ethidium bromide (Thermo Fischer Scientific Inc. Reinach, Switzerland) and visualized by UV light. For DNA sequencing, PCR products were purified (QIAquick® PCR Purification Kit, Qiagen GmbH, Hilden, Germany) and subsequently sent with premixed primers to Microsynth AG (Balgach, Switzerland). The analyzed DNA was diluted in a standard concentration of 22.5 ng each 100 bp fragment length.

4 Results

4.1.1 Genotyping methods

By Real-Time PCR 29 positive cases of *M. tuberculosis* complex were detected.
In culture, *M. bovis* and *M. caprae* were isolated in 17 respectively in 7 lymph node pools out of 528 samples on Mycobacteria Growth Indicator (BD BACTEC™ MGIT™, Becton and Dickinson Company, Franklin Lakes, New Jersey, United States**)**. Species determination was performed using GenoType® MTBC kit (HainLifescience GmbH, Nehren, Germany). Three isolates, two *M. caprae* and one *M. bovis*, grown on liquid culture MGIT™ could not be isolated on solid medium Stonebrink (BBL™Stonebrink™ TB Medium, Becton and Dickinson Company, Franklin Lakes, New Jersey, United States). Furthermore, only by thirteen out of the twenty-four isolates, typical growth of acid-fast bacilli could be observed on Löwenstein-Jensen (Becton and Dickinson Company, Franklin Lakes, New Jersey, United States).

In addition, five samples positive for *M. tuberculosis* complex by direct Real-Time PCR, were culture negative in all selective media used. Hence these samples could not be submitted for MIRU-VNTR analysis. On the other hand, four lymph nodes pools tested negative by Real-Time PCR resulted positive in culture (**Table 3**). Those four samples could furthermore be defined as three *M. bovis* and one *M. caprae* by genotyping using GenoType® MTBC kit (HainLifescience GmbH, Nehren, Germany) and included for MIRU-VNTR analysis.

Table 3. Overview of the samples tested positive at the Institute of Veterinary Bacteriology from March 2013 until November 2014.

Canton	Farm	Isolate	Sample Material	Real-time PCR	Culture	HAIN Genotype
FRIBOURG	A	20593	5 Lymph nodes - Pools	positive (Ct 28.5)	positive	*M. bovis*
		20594	4 Lymph nodes - Pools	positive (Ct 36.3)	positive	*M. bovis*
		20595	4 Lymph nodes - Pools	positive (Ct 24.8)	**No-growth**	
		20596	4 Lymph nodes - Pools	positive (Ct 32.8)	positive	*M. bovis*
		20597	4 Lymph nodes - Pools	positive (Ct 24.3)	positive	*M. bovis*
		20599	4 Lymph nodes - Pools	positive (Ct 32.8)	positive	*M. bovis*
		20600	3 Lymph nodes - Pools	positive (Ct 31.5)	positive	*M. bovis*
	B	13-527	2 Lymph nodes - Pools	**negative**	positive	*M. bovis*
		22667	2 Lymph nodes - Pools	**negative**	positive	*M. bovis*
	C	20665	Caseous liver abscess & 2 Lymph nodes	Liver: positive	positive	*M. bovis*
	D	20175	Caseous lung abscess	positive	positive	*M. bovis*
	E	21113	2 Lymph nodes - Pools	positive (Ct 24.3)	**No-growth**	
	F	22218	2 Lymph nodes - Pools	positive (Ct 37.3)	**No-growth**	
VAUD	G	20606	2 Lymph nodes - Pools	positive (Ct 20.6)	positive	*M. bovis*
		20608	Suspicious tissue lesions	positive (Ct 25.9)	positive	*M. bovis*
		20609	Suspicious tissue lesions	positive (Ct 32.6)	positive	*M. bovis*
		20948	Suspicious tissue lesions	positive (Ct 36.2)	**No-growth**	
		20531	2 Lymph nodes - Pools	**2x negative**	positive	*M. bovis*
		22539	1 Lymph node	positive (Ct 31.2)	positive	*M. bovis*
	H	20632	Caseous lung abscess & 2 Lymph nodes	Lung: positive	positive	*M. bovis*
		20482	2 Lymph nodes - Pools	positive (Ct 36.2)	positive	*M. bovis*
VS	I	20626	2 Lymph nodes & granulomatous lung	Lung: positive (Ct 34.0)	**No-growth**	
AR	J	22848	Liver & lung tissue	positive (Ct 25.9)	positive	*M. caprae*
		22966	3 Lymph nodes - Pools	positive (Ct 32.6)	positive	*M. caprae*
		22971	2 Lymph nodes - Pools	positive (Ct 36.2)	positive	*M. caprae*
		13-450	3 Lymph nodes - Pools	**negative**	positive	*M. caprae*
SG	K	22914	2 Lymph nodes - Pools	positive (Ct 28.5)	positive	*M. caprae*
	L	14-13	2 Lymph nodes - Pools	positive	positive	*M. caprae*
TG	M	13-162	2 Lymph nodes - Pools	positive (Ct 25.5)	positive	*M. caprae*

Reversed line blotting

In total, thirteen *M. bovis* and two *M. caprae* isolates could be confirmed using GenoType® MTBC kit directly from lymph nodes homogenizate (HainLifescience GmbH, Nehren, Germany). Afterwards, all positive cultures were successfully tested by reversed line blotting.

Spoligotyping

Spoligotype analysis revealed a SB0120 profile for the 5 tested *M. bovis* and SB0418 for the 2 *M. caprae* isolates. Spoligotype SB0120 is characterized by the absence of spacers 3, 9, 16, and 39–43; SB0418 by the lack of spacers 1, 3–16, 28, and 39–43.

4.2 MIRU-VNTR analysis

Culture-confirmed bTB cases from March 2013 to January 2014 were included for MIRU-VNTR analysis. A PCR product was obtained for the complete set of tested MIRU-VNTR loci using the panel of 17 *M. bovis*, 7 *M. caprae* and 3 *M. tuberculosis* complex reference strains. Typability was therefore 100% for the 49 chosen markers. Two clusters of profiles (one for *M. bovis*, one for *M. caprae*) were detected for the Swiss isolates among the analyzed loci (**Table 4 and 5**). In both cases single locus variation (SLV) could be found in four *M. bovis* isolates out of seventeen and in one *M. caprae* isolate out of seven (**Table 6 and 7**).

Table 4. Allele profiles of the Swiss *M. bovis* and *M. caprae* isolated in 2013 and 2014 compared with three reference strains on the 24 MIRU-VNTR standard panel. Nomenclature in accordance with the European Union Reference Laboratory for Bovine Tuberculosis, VISAVET Health Surveillance Centre, Complutense University of Madrid.

	MIRU 02	Mtub04	ETRC	MIRU 04	MIRU 40	MIRU 10	MIRU 16	Mtub21	MIRU 20	QUB11b	ETRA	Mtub29	Mtub30	ETRB	MIRU 23	MIRU 24	MIRU 26	MIRU 27	Mtub34	MIRU 31	Mtub39	QUB26	QUB4156	MIRU 39
	154	424	577	580	802	960	1644	1955	2059	2163b	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348
M. tuberculosis H37Rv	2	2	3	3'	1	3	2	2	2	5	3	4	2	3	6	1	3	3	3	3	5	5	2	2
Swiss field strain *M.caprae*	2	4	4	2	2	6	4	2	2	5	5	3	4	3	4	2	4	3	2	3	1	3	3	2
Swiss field strain *M. bovis*	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
M. bovis BCG Pasteur ATCC 35734™	2	0	5	2'	2	2	3	1	2	3	5	2	2	5	4	2	5	3	3	3	2	5	0	2
M. bovis BCG Tice ATCC 27289™	2	0	4	1'	3	2	3	1	2	3	5	2	2	5	4	2	5	3	3	3	2	5	0	2

Table 5. Allele profiles of the Swiss *M. bovis* and *M. caprae* isolated in 2013 and 2014 compared with three reference strains analysed with the 25 MIRU-VNTR additional loci. Nomenclature in accordance with the European Union Reference Laboratory for Bovine Tuberculosis, VISAVET Health Surveillance Centre, Complutense University of Madrid.

					QUB1281c								QUB11a					QUB3232								
	79	569	917	1121	1281c	1305	1443	1895	1907	1982a	1982	2074	2163a	2372	2705	3155	3189	3232	3239	3291	3351	3594	3820g	4120	4155g
M. tuberculosis H37Rv	6	2	2	4	2	2	1	4	2	2	5	3	2	2	1	3	2	3	2	1	2	3	3	2	2
Swiss field strain *M.caprae*	3	0	1	3	2	1	2	4	1	5	3	1	8	1	1	3	2	11	1	2	2	1	7	2	5
Swiss field strain *M. bovis*	8	1	2	3	2	1	2	4	1	4	3	2	10	1	2	3	2	6	1	4	2	3	11	2	7
M. bovis BCG Pasteur ATCC 35734™	10	1	2	3	2	1	2	4	1	3	3	2	10	1	2	3	2	5	1	4	2	3	7	2	11
M. bovis BCG Tice ATCC 27289™	10	1	2	3	2	1	2	4	1	3	3	2	10	1	2	3	2	5	1	4	2	3	7	2	11

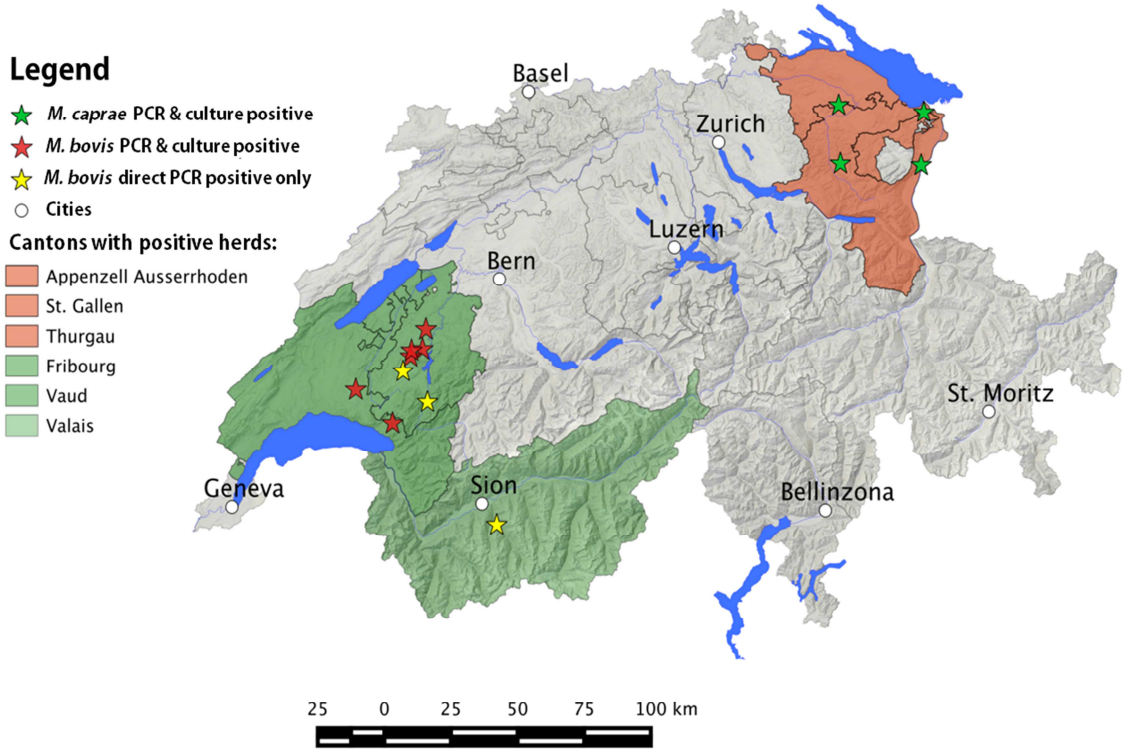

Figure 3. Map of Switzerland showing the geographical origin of the positive samples. Green stars for *M. caprae*, red stars for *M. bovis*, both positive by direct PCR testing and successively in culture; yellow stars for *M. bovis* positive only by direct PCR testing and culture negative. The Swiss Cantons where cases of bTB were detected are elucidated. Main Swiss lakes and rivers are marked in blue.

4.2.1 *Mycobacterium bovis*

The seventeen *M. bovis* isolates isolated by culture and analyzed by MIRU-VNTR were collected from six farms, all of them located in two Cantons: Fribourg (4 farms, 10 isolates), Vaud (2 farms, 7 isolates) as shown on **Figure 3**. Thirteen isolates shared a common VNTR profile (**Table 6**); three showed a SLV in locus VNTR 2059 (alias MIRU 20) with the loss of one of the three repeat units and one isolate showed a double band profile in the described locus.

Table 6. Allele profiles of the seventeen Swiss *M. bovis* isolates analyzed. The SLVs and the double allele are highlighted in yellow and orange respectively.

Farm	Isolate	MIRU 02	Mtub04	ETRC	MIRU 04	MIRU 40	MIRU 10	MIRU 16	Mtub21	MIRU 20	QUB11b	ETRA	Mtub29	Mtub30	ETRB	MIRU 23	MIRU 24	MIRU 26	MIRU 27	Mtub34	MIRU 31	Mtub39	QUB26	QUB4156	MIRU 39
		154	424	577	580	802	960	1644	1955	2059	2163b	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348
A	20593	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20594	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20596	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20597	2	0	4	3	2	2	3	1	2	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20599	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20600	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	**2**	5	1	3	3	2	5	1	2
B	13-527	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	22667	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
C	20665	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
D	20175	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
G	20606	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20608	2	0	4	3	2	2	3	1	2+3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20609	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20531	2	0	4	3	2	2	3	1	2	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	22539	2	0	4	3	2	2	3	1	2	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
H	20632	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2
	20482	2	0	4	3	2	2	3	1	3	3	4	3	4	7	4	2	5	1	3	3	2	5	1	2

In order to prove the presence of two clonal variants in isolate 20608 we obtained twelve series of subcultures from the original mycobacterial colonies growth in the Mycobacteria Growth Indicator Tube (BD BACTEC™ MGIT™ Becton and Dickinson Company, Franklin Lakes, New Jersey, United States**).** In **Figures 4 to 7** the electropherograms of subculture I, III, VII and XII for the locus 2059 are shown. Notable is the decline of the PCR product of 673 (± 1) bp corresponding to allele 3, meaning that in subculture I, III, and VII the clonal population showing this allele was decreasing. In **Figure 7**, the PCR product from subculture XII is displayed, the amplicon corresponding to allele 3 is completely absent.

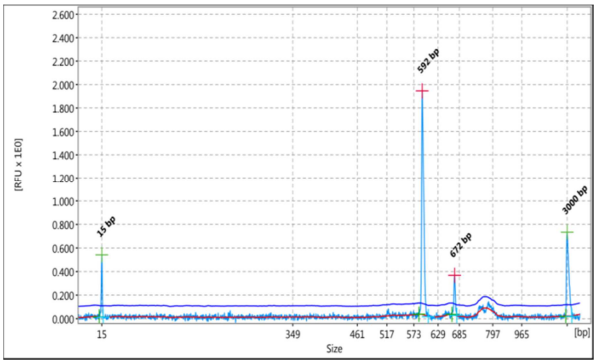

Figure 4. Electropherogram of subculture I.

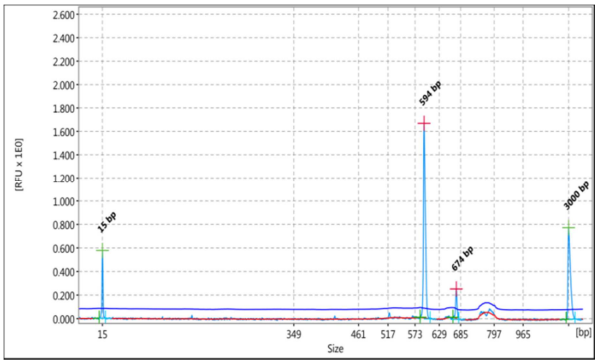

Figure 5. Electropherogram of subculture III.

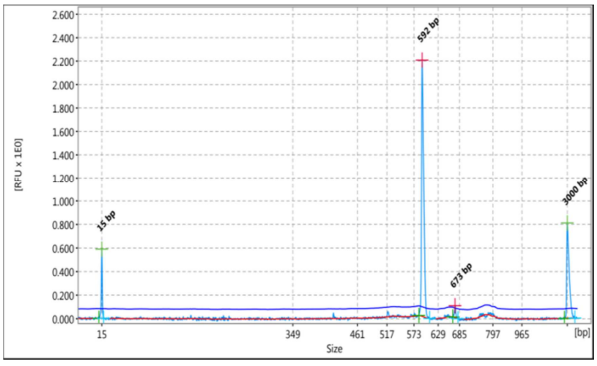

Figure 6. Electropherogram of subculture VII.

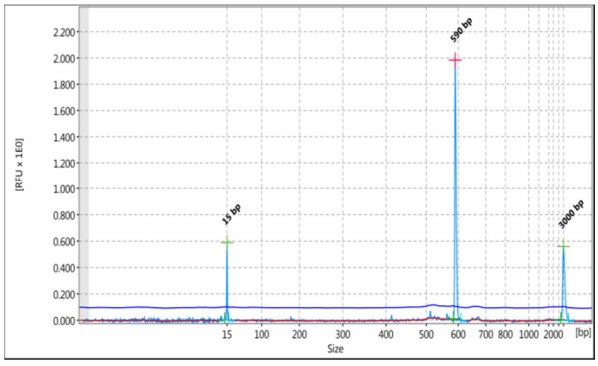

Figure 7. Electropherogram of subculture XII.

In order to exclude a mutation other than the loss of one repeat unit, e.g. a deletion in the flanking region, we opted for sequencing of the two bands.
The results of the sequenced fragments are shown in the following illustrations (**Figure 8-9**):

Figure 8. Comparison of the sequenced *M. bovis* 20608 (long fragment) with another isolate from the same farm (20609). For the sake of completeness the sequence of *M. tuberculosis* H37Rv showing only two repeat units is aligned in the third line. Forward and reverse primers of locus 2059 are annotated in purple, the consensus repeat units in green.

Figure 9. Comparison of the sequenced *M. bovis* 20608 (short fragment) with another isolate from the same farm (22539). The sequence of *M. tuberculosis* H37Rv is aligned in the third line. Forward and reverse primers of locus 2059 are annotated in purple, the consensus repeat units in green.

From **Figures 8** and **9** it can be recognized how the two sequences displayed by isolate 20608 correspond to an allele 3 respectively 2, clustering perfectly with the two variants present in farm G.

4.2.2 *Mycobacterium caprae*

The seven *M. caprae* isolates originated from four farms, located in three Cantons: Appenzell Ausserrhoden (1 farm, 4 isolates), St. Gallen (2 farms, 2 isolates) and Thurgau (1 farm, 1 isolates) as shown in **Figure 3**. All the samples exhibited exactly the same MIRU-VNTR profile revealing an infection with a clonal population of *M. caprae*. As shown in **Table 7** the only exception was isolate 13-450 from Appenzell Ausserrhoden, displaying a double allele in locus VNTR 802 (alias MIRU 40).

Table 7. Allele profiles of the seven Swiss *M. caprae* isolates analyzed. The double allele in locus VNTR 802 is highlighted in orange.

Farm	Isolate	MIRU 02	Mtub04	ETRC	MIRU 04	MIRU 40	MIRU 10	MIRU 16	Mtub21	MIRU 20	QUB11b	ETRA	Mtub29	Mtub30	ETRB	MIRU 23	MIRU 24	MIRU 26	MIRU 27	Mtub34	MIRU 31	Mtub39	QUB26	QUB4156	MIRU 39
		154	424	577	580	802	960	1644	1955	2059	2163b	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348
J	22848	2	4	4	2	2	6	4	2	2	5	5	3	4	3	4	2	4	3	2	3	1	3	3	2
J	22966	2	4	4	2	2	6	4	2	2	5	5	3	4	3	4	2	4	3	2	3	1	3	3	2
J	22971	2	4	4	2	2	6	4	2	2	5	5	3	4	3	4	2	4	3	2	3	1	3	3	2
J	13-450	2	4	4	2	0+2	6	4	2	2	5	5	3	4	3	4	2	4	3	2	3	1	3	3	2
K	22914	2	4	4	2	2	6	4	2	2	5	5	3	4	3	4	2	4	3	2	3	1	3	3	2
L	14-13	2	4	4	2	2	6	4	2	2	5	5	3	4	3	4	2	4	3	2	3	1	3	3	2
M	13-162	2	4	4	2	2	6	4	2	2	5	5	3	4	3	4	2	4	3	2	3	1	3	3	2

While the larger fragment of 13-450 corresponded to an allele 2, reflecting the result of the other tested isolates, the size of the smaller PCR product was compatible with an allele 0 as shown in Figures 10 and 11.

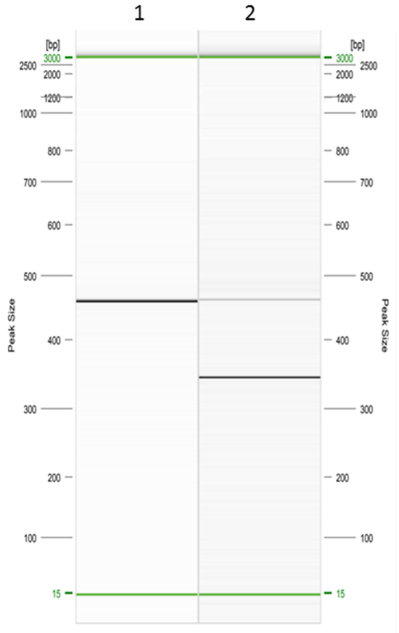

Figure 10. Virtual gel image visualized by the QIAxcel ScreenGel software.
Lane 1: *M. caprae* 22971; lane 2: *M. caprae* 13-450.
PCR products of locus VNTR 802 are shown in form of black bands. Green displayed the alignment marker (QX 15 bp – 3 Kb).

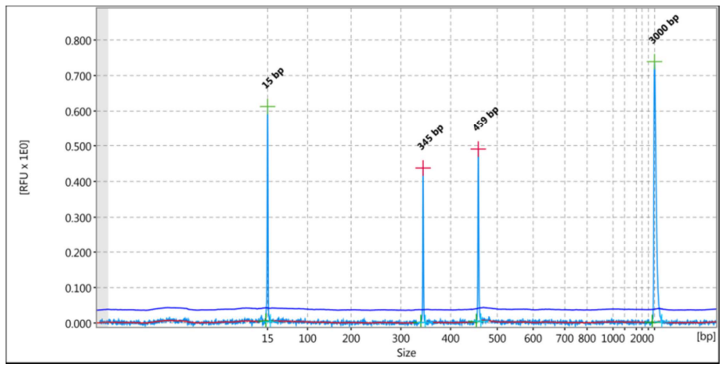

Figure 11. PCR products of isolate *M. caprae* 13-450 in locus VNTR 802 are shown in form of electropherogram.
Left and right the peaks of the alignment marker (15 bp – 3 Kb) are displayed, in the middle the two bands corresponding to different allele.

of the two bands was performed. Contrary to what we expected, in addition to the loss of the two 54 bp long repeat units, 9 nucleotides of its flanking sequence are absent in the smaller PCR product. Furthermore, as shown in Figure 13, a total of 30 single-base mutations could be distinguished. In order to exclude amplification mismatch due to the PCR process, MIRU-VNTR analysis and sequencing where repeated twice and lead to identical findings.

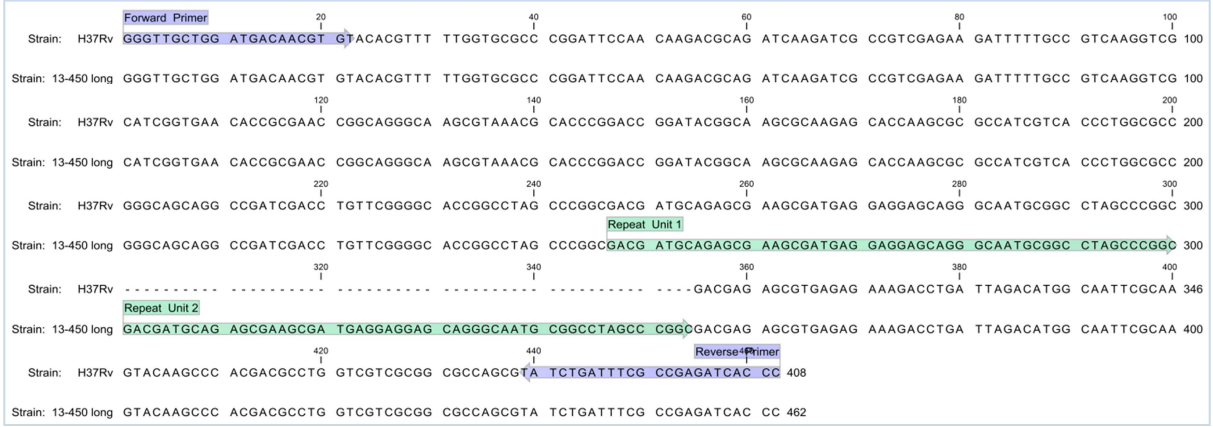

Figure 12. Comparison of the sequenced *M. caprae* 13-450 (long fragment) with *M. tuberculosis* H37Rv. The two 54 bp long repeat units are labeled in green, H37Rv exhibit only one repeat unit in this MIRU-VNTR locus. Forward and reverse primers are annotated in purple.

Figure 13. Comparison of the sequenced *M. caprae* 13-450 (short fragment) with *M. tuberculosis* H37Rv. The two 54 bp long repeat units and 9 nucleotides of its flanking sequence are absent from this amplicon. Furthermore, numerous single-base mutations could be distinguished in red. Forward and reverse primers are annotated in purple.

4.2.3 Difficulties encountered during the establishment of high-throughput QIAxcel System

As previously described [60, 61], the QIAxcel advanced System is highly accurate in sizing PCR products up to 600 bp (+ 0 - 10 bp) in comparison to conventional agarose gel electrophoresis (**Figures 14a-b**).

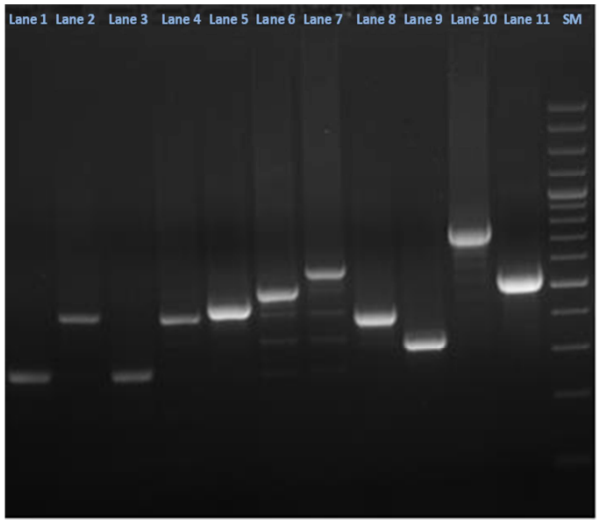

Figure 14a. Conventional agarose gel.

Four different mycobacterial strains isolated in Switzerland were analyzed in three core gene loci (lane 1-4, lane 5-8, lane 9-11).
The PCR products were visualized on a 2% agarose gel.
SM: 100 bp DNA size marker.

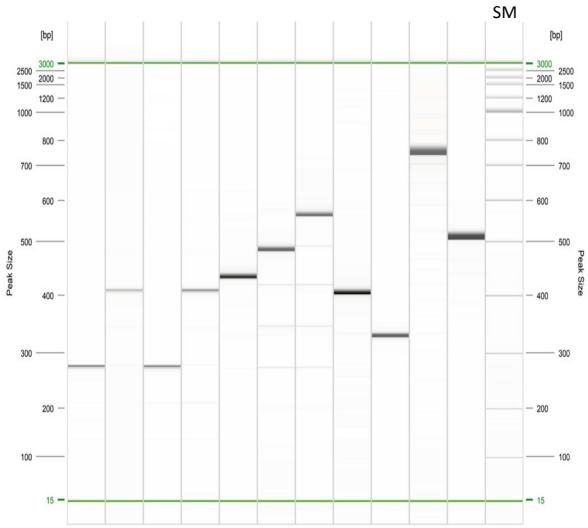

Figure 14b. Native capillary electrophoresis.

The same PCR products as in **figure 14a** were analyzed using the QIAxcel Advanced System.

SM: QX DNA Size Marker 100 bp – 2.5 kb.

However, we encountered some problems sizing larger fragments, in particular for the loci 3232, 2163a, 3820 and 4155. In those cases the expected allelic code obtained by sequencing could not be confirmed by the QIAxcel analysis.

Therefore we tried to minimize the size of the amplicons by setting new primer pairs closer to the repeat units. The described changes solved the discrepancy problems in two cases, namely in locus 3820 and 4155, where we could shorten the size of allele 0 of 134 and 531 bp respectively, without consequences to the PCR reproducibility.

Table 8. Comparison of fragment size obtained by sequencing and average size by QIAxcel capillary electrophoresis after PCR amplification of locus VNTR 3232. Highly accurate sizing up to 600 bp fragments and overestimation of longer amplicons has been observed.

Tested Strain	Fragment size by sequencing (bp)	Fragment size by QIAxcel (bp)
***M. tuberculosis* H37Rv**	**349**	**349**
***M. bovis* BCG Tice**	**461**	**465**
22667 *M. bovis*	**517**	**519**
IMM *M. bovis*	**629**	**642**
22848 *M. caprae*	**797**	**856**
22928 *M. microti*	**965**	**1084**

However, we did not obtain accurate results for the *M. caprae* and *M. microti* isolates tested in locus 3232 (**Table 8**) and for the *M. bovis* and *M. microti* isolates tested in locus 2163a (data not shown). For this reason we have developed a size marker, or allelic ladder, specific for each of the above described loci, composed of amplicons of known size with different repeat unit numbers as shown in the electropherograms in **Figures 15a** and **15b**.

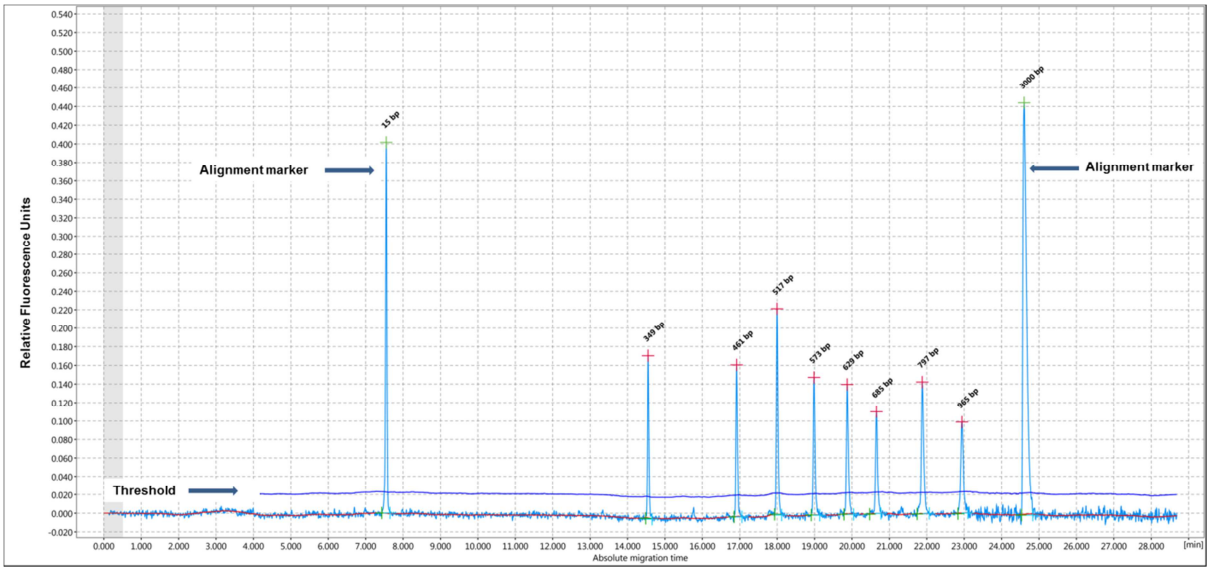

Figure 15a. Allelic ladder specific for locus VNTR 3232. Eight amplicons of different length are used as reference size markers.

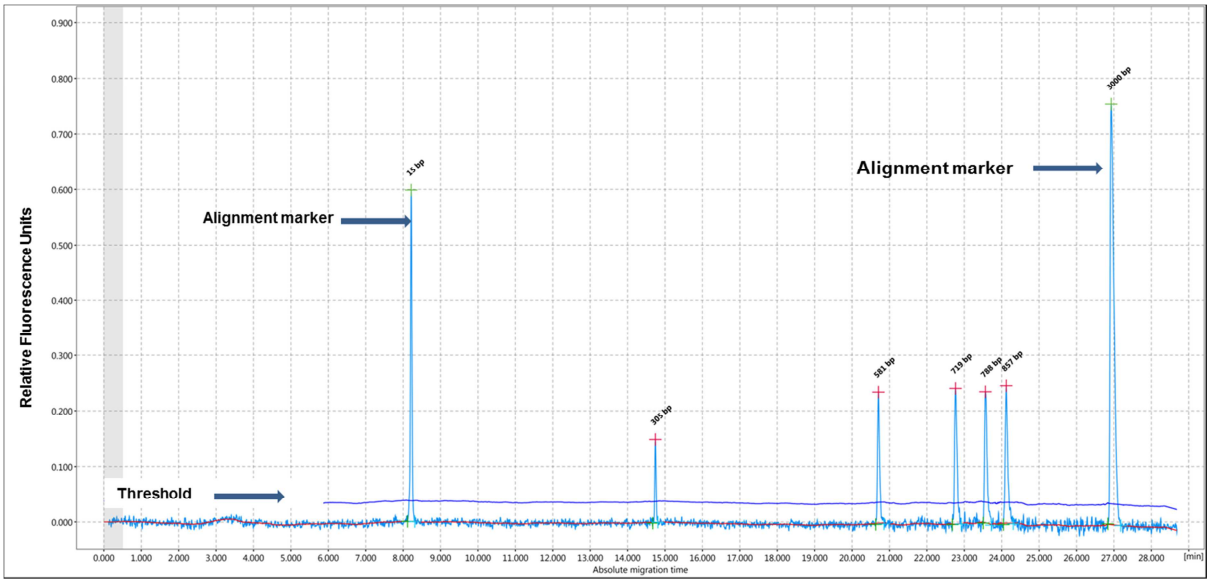

Figure 15b. Allelic ladder specific for locus VNTR 2163a. Five amplicons of different length are used as reference size markers.

Allelic ladders represent a satisfactory solution for the problem of overestimation error due to the different nucleotide sequences between size marker and analyzed fragment. In fact, similar to conventional agarose gel electrophoresis, DNA migration in capillary electrophoresis seems to be dependent additionally upon its nucleotide sequence and not only upon the length of the fragment as previously described. The final concentration of each allelic ladder was 10 ng /µl as suggested by Qiagen for ready-to-use size markers.

5 Discussion

The epidemiological settings of the present two outbreaks of bovine tuberculosis in Switzerland, and the genotyping findings strongly support the hypothesis that the observed positive cases result from two single clonal populations of aetiological strains. The analyzed isolates showed the same spoligotype pattern, SB0120 for *M. bovis* hailing from the western part of the country and SB0418 for *M. caprae* responsible for the second outbreak in the eastern part of Switzerland.

5.1 Allelic variability

As previously described, 13 out of 17 *M. bovis* isolates shared an identical MIRU-VNTR profile (**Table 6**) in all the 49 loci used for genotyping. Three isolates showed a single locus variation (SLV) in locus VNTR 2059 (alias MIRU 20) with the loss of one of the three repeat units, and one isolate showed a double band profile in the described locus. Therefore the question about a possible heterogeneity of the isolated mycobacteria and multiple sources of infection arose.
Assuming the very low prevalence of bovine tuberculosis pathogens in the Swiss cattle population and the high similarity of the obtained MIRU-VNTR profiles, it can be postulated that the two patterns observed in isolate 20608 are not deriving from two independent strains but, on the contrary, resulting from the same ancestor strain. Epidemiologically we could not prove that the microevolution occurred in patient 20608, since animals from farm A and G were regularly in contact, spending the summer months on the same pasture, and in both holdings the two patterns were observed.
Single locus variation or double locus variation (DLV) is a well-known phenomenon for *M. tuberculosis* [44, 62] as well as for *M. bovis* [41, 46, 50]. However, the simultaneous detection of both allele variants is to date a rarity.
In contrast to a mixed infection, defined as the simultaneous infection by two or more *M. bovis* strains with evident distinct DNA fingerprints, in our case, patient 20608 was infected with a bacterial subpopulation. This phenomenon is rare but not unique and it has been widely described in human patients infected with *M. tuberculosis*. Many authors agree that it might be due to the gradual evolutionary events that occur post-infection, resulting from the interactions with the new host [63-65].
Another factor thought to be cause of variability in VNTR sequences is the slipped-strand mispairing (SSM), which may occur due to incorrect base pairing in regions of repetitive DNA during replication. This phenomenon, in combination with an inadequate DNA mismatch repair system, can lead to deletions or insertions of one or more repeat units (**Figure 16**).

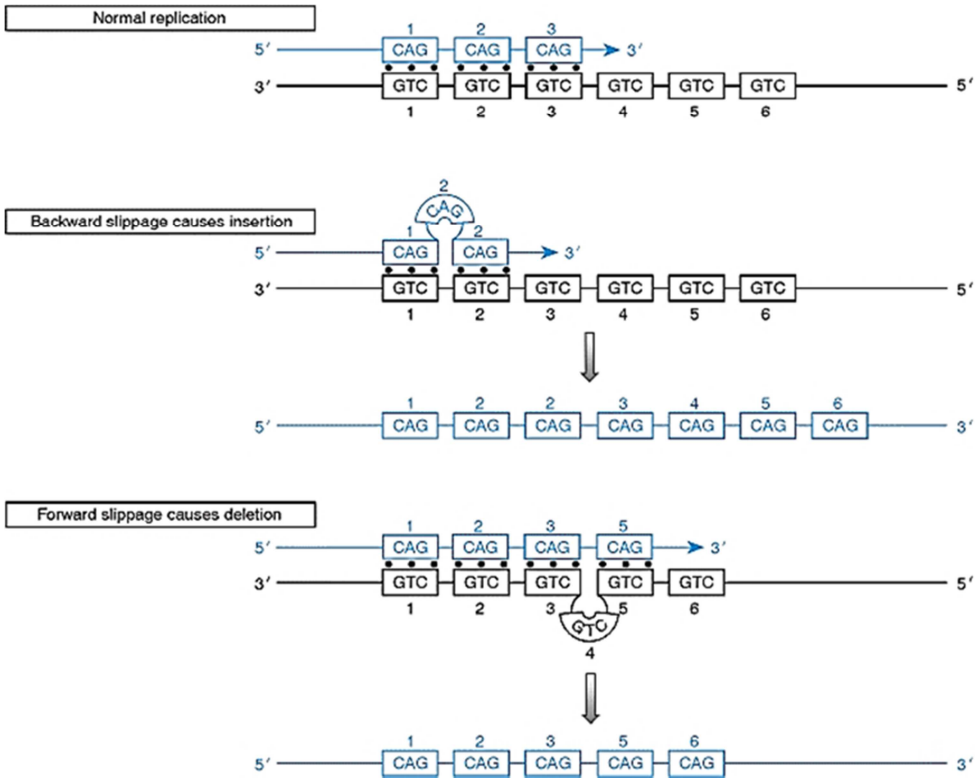

Figure 16. Schematic representation of slipped-strand mispairing (SSM) during DNA replication.
The number of short repeat units in the newly synthesized DNA strand (light blue) do not correspond with the one of the parental strand. Insertion or deletion can lead to variability in the number of repeat units [66].

Interestingly, in contrast to the literature concerning the well-documented *M. tuberculosis*, variability was detected only among the standard panel of 24 loci and not in the commonly called hypervariable loci VNTR 1982, VNTR 3232, VNTR 3820 or VNTR 4120. This confirms the widely accepted concept that, depending on the regional evolution of the *M. tuberculosis* complex agents, a different set of MIRU-VNTR should be selected and adopted. The variability observed in *M. bovis* for locus VNTR 2059 in this study, despite the small number of analyzed isolates, suggests a possible remarkable discriminatory power for the mentioned locus.

In contrast, the same marker has been defined as poorly discriminatory in many studies, and it is not one of the eight loci recommended by the EURL for analysis of *M. bovis* strains [47-49]. Another example from the literature is VNTR 2163a (QUB11a), showing a low discriminatory value in *M. bovis* isolated from the Donana national park [50], whereas in other cases as in Italy [49], Portugal [67] and Ireland [48] a high degree of discrimination has been observed.

Romero *et al.* have described the first co-infection of different *M. bovis* strains in animals. MIRU-VNTR analysis provides a suitable genotyping tool to detect mix-infections and furthermore allowing strain discrimination [50]. Since Swiss isolates showed high similarity but were not identical, we suggest that the same *M. bovis* strain has been circulating in Switzerland, and more precisely in Canton Fribourg, for many years. This suggestion could be validated by a clustering isolate obtained from an archival specimen originating from the same region. In fact, over the course of the present study we had the chance to analyze an isolate derived from a cow slaughtered in 1998.

Interestingly, the obtained DNA clustered in all the 49 tested loci with the most common *M. bovis* strain of the present outbreak. The origin of this animal was farm A. Whether such an ancestral strain

persisted in the region since many decades or had been imported into the country at a modern time is a yet unanswered question. However, the presence of *M. bovis* among the bovine population in a subclinical form can be postulated, since the import of an animal infected with a pathogen sharing the same MIRU-VNTR profile is to be considered unlikely. In fact the high discriminatory power of the MIRU-VNTR genotyping technique has been largely described by different authors in many different settings [49, 67, 68]. Moreover the persistence of SB0120 in Canton Fribourg for at least 15 years could explain the SLV, a time span reasonably long enough to permit genomic variations (see above).
In order to clarify the provenience of the ancestral strain, some investigations in collaboration with bordering countries have been performed. *M. bovis* SB0120, also known as BCG-like, is an often isolated spoligotype in European cattle populations: the most frequent type observed in Italy and France, with 54% and 26% prevalence respectively [49, 69], and reported as highly prevalent in Belgium, Germany, Netherlands, and Spain. Additionally it has been described in extra-European countries, too: Tunisia 45% [70], Iran [71], Argentina, and Brazil. To date, it has not been isolated in Great Britain [9]. Epidemiologically, the spoligotyping technique was thus not informative.

In contrast, comparing the obtained MIRU-VNTR allele profile with isolates from central Europe an interesting finding could be ascertained. In France the genotyping analysis of *M. bovis* is based on eight highly discriminatory markers, namely VNTR 580, VNTR 577, VNTR 2165, VNTR 2461, VNTR 3232, VNTR 2163a, VNTR 2163b, and VNTR 4052. Limiting the comparison to these eight loci, one clustering profile could be found in the French database, a *M. bovis* SB0120 strain isolated from a cat in 1989 and hailing from a central region in France. This MIRU-VNTR profile could not be observed in any other isolates, even if other spoligotype patterns were taken into account. Unfortunately the attempt made to trace back the exact provenience of the cat resulted vain. The majority of the more than thousand French SB0120 strains exhibit allele 4, 5, and 6 in locus 2461, while the Swiss *M. bovis* and the French cat from 1989 show a 7. Omitting marker 2461, two other strains appertaining to the widespread family of the Alpine strains match the seven loci tested in France, some of them hailing from regions very close to the origin of the Swiss outbreak, like Haute-Savoie, Savoie and Isère. Therefore it could be postulated that as a result of a SLV a new profile originated from a clonal infection chain has been circulating in the bordering region between France and Switzerland since the end of the last century (Maria Laura Boschiroli, personal communication).

5.2 Situation in the eastern part of Switzerland

M. caprae SB0418 has been described for the first time in Belgium. Meanwhile, analogous to *M. bovis* SB0120, the pathogen has been reported in several countries, and in central Europe more than 50% of the isolates correspond to the described spoligotype [69, 72]. Epidemiologically, the spoligotyping thechnique is thus not informative. On the other hand, the analyzed isolates shared an identical discriminatory MIRU-VNTR profile with an endemic Austrian strain known as “Lechtal” type. In Switzerland, the tradition of pasture on pre-alpine and alpine regions during the summer months remains very common. Often herds from different farms share the same fields and it is not rare to observe Swiss livestock grazing in bordering countries were bTB has been documented both in cattle and in wildlife [18]. This is for example the case for the west Austrian regions of Vorarlberg and Tyrol, bordering the Swiss Cantons of St. Gallen and Grisons. In the autumn these herds return to their farms of origin and it is not uncommon that single animals are sold to other holdings. Such cattle movements become problematic when bTB outbreaks are uncovered, forcing inter-cantonal, or even nationwide control programs. A clear correlation between the summer pasturing regions and the affected farms has thus been confirmed. A question that remains open is the precise source of infection, which could be either infected Austrian domestic ruminants or wild animals.

Since the beginning of the current century, cattle from the Austrian regions of Vorarlberg and Tyrol have shown gross lesions at meat-inspection due to bTB. In parallel, since 1999 the Austrian Agency for Health and Food Safety (AGES) reported sporadic cases of tuberculosis in free-ranging red deer within the same provinces. Considering that direct contact is not necessary for the transmission of the pathogen, grazing cattle may be exposed to high risk of infection pasturing fields contaminated by excretions such as saliva, feces, urine or discharged pus.
Contrary to previous assumptions, a recent study performed under natural weather conditions demonstrated the long persistence of infectious *M. bovis* in the environment, such as water and hay (up to 58 days), strongly suggesting that indirect transmission could play an important role in bTB infection chains [73]. For this reason, in modern days, wildlife reservoirs are considered to play a crucial role in eradication programs of bTB and represent an important challenge in the elimination of this zoonosis. An ongoing surveillance project started by the Swiss Federal Food Safety and Veterinary Office (FSVO) will propose an outlook about the prevalence of bTB pathogens in wild ungulates residing in the Eastern part of Switzerland.
Interestingly the SLV observed in isolate 13-450 resulted from the loss of two repeat units in addition to numerous single-base mutations randomly distributed throughout the flanking region of the VNTR locus (**Figure 13**). This phenomenon has been noticed by Roring *et al.* in *M. bovis* field isolates from Northern Ireland and the Republic of Ireland, where heterogeneity in the nucleotide sequence of different isolates has been described [41]. To the author's knowledge such sequence degeneracy has never been reported before in the *M. caprae* species and further research is needed to identify and understand the mechanisms leading to such genomic variations.

5.3 Weakness of this study

A certain weakness of this study was that spoligotyping has been conducted only on a few isolates; we assumed that testing some isolates representative for the most afflicted farm was sufficient. It is important to notice that many authors agree with the fact that MIRU-VNTR has a higher discriminatory power compared to spoligotyping. However, it should be kept in mind that in some rare cases, it has been possible to detect genetical variability only through different markers [62].

5.4 Significance of the obtained results

This study represents the first MIRU-VNTR analysis on bovine field samples from Switzerland. It provides a robust tool to differentiate epidemiologically disparate isolates, and with the further establishment of a European MIRU-VNTR database for *M. bovis*, it will be possible to trace back the most important strains. The initial intent of determining the discriminatory power of the different loci testing Swiss field isolates could not be carried out because of the low number of mycobacterial isolates and the lack of variability.

The performed MIRU-VNTR analysis has been enabled by the use of the QIAxcel Advanced System (Qiagen GmbH, Hilden, Germany). QIAxcel is an automated capillary electrophoresis instrument that can deal with up to 96 samples per run. The shortest turnaround time for one run of 12 specimens using a high resolution cartridge is approximatively 33 minutes.
This represents a notable time-saving tool compared with the conventional pulsed-field agarose technique. The results can successively be exported in either a gel-view format (**Figure 10**) or

electropherograms (**Figure 11**). We established optimal and reproducible PCR conditions combined with an automated peak-calling-based fragment analysis. We obviated the unsatisfactory accuracy of the QIAxcel instrument sizing fragments larger than 800 bp through the development of an allelic ladder specific for the hypervariable loci 3232 and 2163a. As previously described by others, some loci showed an unsatisfactory PCR amplification for *M. bovis* and *M. caprae* strains [46]. We observed this phenomenon for the loci VNTR 3336, VNTR 0024, VNTR 3336, VNTR 2990, VNTR 1612 and QUB 1451. Therefore we decided not to include these loci into our analysis panel. A notable increase of the amount of PCR product and reproducibility was noticed by adding Q-Solution (Qiagen, Hilden, Germany), known to be favorable for high GC fragments as mycobacterial ones (genomic GC% = 65%). Since a panel of 49 markers represents a time- and cost-intensive effort that could not be performed for routinely intended analysis, further ampler investigations are needed to establish a discriminatory set of 10–15 markers specifically indicated for bTB agents possibly isolated in Switzerland in the future.
As supposed by many authors [46-50, 68], the high variability of the *M. bovis* population impede the international utilization of a consensus set of markers that provides a satisfactory discriminatory power in any situation. Due to the very low prevalence of bTB in Switzerland, an analysis of the discriminatory power of the different loci could not be assessed. This study revealed the presence of one single strain of *M. bovis* and of *M. caprae* on the territory. For this reason a standardized panel of loci, not exceeding ten loci, that allows an appropriate genotyping of Swiss isolates is still requested.

During the establishment of the MIRU-VNTR technique, we have been confronted with the definition of the peak calling allele function of the QIAxcel ScreenGel software. Additionally to the different names of some MIRU-VNTR loci described by some authors, e.g. ETR E in [38], also known as MIRU 31 in [39] alias VNTR 3192 [35], we spotted different allele coding systems. In other words, a specific reference strain, whether H37Rv or *M. bovis* BCG Pasteur is described with a different allele profile for the same locus. This discrepancy in the allele definition between different studies, as explained by Skuce and others [42], is due in part to the fact that many MIRU-VNTR loci contain partial repeat sequences that can represent a consistent proportion of the entire repeat.

In fact, for some loci the allele naming was defined in different ways by some authors, depending on whether they considered this partial repeat as a whole repeat. We encountered disagreement in locus VNTR 577 (alias ETR C), first described by Frothingham and Meeker-O'Connell [38], where *M. tuberculosis* H37Rv shows an allele 4 (4 repeat units of 58 bp minus 21, i.e. taking into account a partial repeat of 37 bp); Smittipat and Palittapongarnpim [45] on the other hand described the same strain as an allele 3 (3 repeat units of 58 bp plus 21, i.e. not taking into account a partial repeat of 37 bp in the allele count system). The same discordance was encountered in locus MIRU-VNTR 2163a (alias QUB-11a). We decided to adopt the same allele-naming table proposed by the European Union Reference Laboratory for Bovine Tuberculosis, VISAVET Health Surveillance Centre, Complutense University of Madrid. In locus VNTR 577 the adopted nomenclature was first described by Smittipat and Palittapongarnpim [45] and in locus VNTR 2163a by Skuce and others [42]. In both loci the partial repeat units are not taken into account for the allele profile definition.

6 Conclusion

Since molecular techniques have been developed, typing of mycobacteria has become a common practice, especially for members of the *M. tuberculosis* complex. In veterinary medicine, spoligotyping remains the first-line technique for mycobacterial typing, not only because of the straightforward and rapid use but also because of the worldwide standardization allowing information exchanges between laboratories. On the other hand, the MIRU-VNTR technique for *M. bovis* isolates became popular after the proposal of standardization displayed by Allix *et al.* in 2006 [46]. MIRU-VNTR typing is highly discriminative for *M. tuberculosis* and many authors propose that it could be the method of choice for *M. bovis* typing, too. To date, in the epidemiological context MIRU-VNTR is especially indicated in tracing the spread of the pathogen between herds and wild reservoirs. In contrast to other genotyping methods, e.g. IS6110-RFLP typing, MIRU-VNTR can be applied directly from heat-killed mycobacterial cultures as soon as acid-fast bacilli are detected. Therefore, time-consuming intensive mycobacterial culturing can be spared, since a minimal amount of extracted DNA is sufficient to perform an examination. For this reason MLVA provides a rapid epidemiological investigation tool, permitting the tracing of tuberculosis infection chains.

7 References

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8 Acknowledgements

I would like to take this opportunity to thank my major advisor, Professor Dr. Max M. Wittenbrink, for supervising my whole doctoral thesis. Only his disponibility, understanding, guidance and enormous encouragement enabled me to complete this important research project. I wish to thank Professor Dr. Roger Stephan for helpful suggestions and comments of the manuscript.

I am deeply grateful to the entire team of the Institute of Veterinary Bacteriology. They have all contributed with constructive cooperation and collaboration, providing a very motivating working atmosphere. Especially dipl. biol. Simone Scherrer, Ute Friedel and Daniel Frei were three fundamental reference people for their great technical support and valuable advice. I am grateful to Annegret Buff for proofreading the manuscript.

I would also like to thank M. Sc. Mirjana Kozulic for providing indispensable information, support and experience regarding the use of the QIAxcel capillary electrophoresis.

And above all, very special thanks go to Vivien Rampling and to my family, encouraging and supporting me in every difficult choice throughout the years.

This project was founded by the Swiss Federal Food Safety and Veterinary Office.

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